Immunity
Article

Central Role of Defective Interleukin-2 Production in the Triggering of Islet Autoimmune Destruction

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SUMMARY

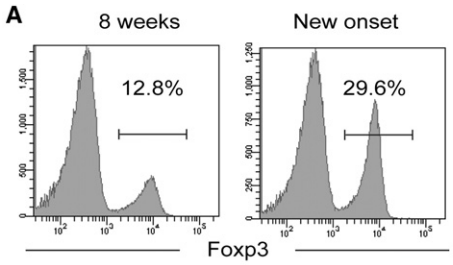
The dynamics of CD4$^+$ effector T cells (Teff cells) and CD4$^+$Foxp3$^+$ regulatory T cells (Treg cells) during diabetes progression in nonobese diabetic mice was investigated to determine whether an imbalance of Treg cells and Teff cells contributes to the development of type 1 diabetes. Our results demonstrated a progressive decrease in the Treg cell:Teff cell ratio in inflamed islets but not in pancreatic lymph nodes. Intra-islet Treg cells expressed reduced amounts of CD25 and Bcl-2, suggesting that their decline was due to increased apoptosis. Additionally, administration of low-dose interleukin-2 (IL-2) promoted Treg cell survival and protected mice from developing diabetes. Together, these results suggest intra-islet Treg cell dysfunction secondary to defective IL-2 production is a root cause of the progressive breakdown of self-tolerance and the development of diabetes in nonobese diabetic mice.

INTRODUCTION

Type 1 diabetes (T1D), also known as autoimmune diabetes, results from autoimmune destruction of insulin-producing β cells in the pancreatic islets of Langerhans. The disease is characterized by a loss of blood-glucose homeostasis accompanied by inflammatory infiltrates in the islets. Both genetic and environmental factors contribute to the development of the disease. Hallmarks of human T1D are observed in the nonobese diabetic (NOD) mouse, a strain identified 27 years ago in an inbred colony in Japan (Makino et al., 1980). NOD mice provide a valuable tool for dissection of the pathogenesis of T1D. Over 20 genetic loci, termed insulin-dependent diabetes (Idd) loci, have been linked to disease susceptibility in both human patients and NOD mice (Todd and Wicker, 2001). Among these genes, those that encode MHC class II, insulin, CTLA-4, and IL-2 have been identified as contributors to the breakdown of central and/or peripheral tolerance, although the precise mechanisms associated with pathogenesis remain to be defined (Chentoufi and Polychronakos, 2002; Nakayama et al., 2005; Prochazka et al., 1987; Todd et al., 1987; Ueda et al., 2003; Yamanouchi et al., 2007).

Circumstantial evidence suggests that regulatory T cells (Treg cells) control the progression of diabetes. Disruption of Treg cell development and homeostasis by blocking of the CD28-B7 pathway or IL-2 activity in NOD mice leads to acceleration of diabetes (Salomon et al., 2000; Setoguchi et al., 2005). In addition, *Tcrα*$^{-/-}$-deficient, *Rag1*$^{-/-}$-deficient, or *Foxp3*-deficient scurfy BDC2.5 T cell receptor (TCR)-transgenic mice, which are completely devoid of Treg cells, display no delay between the onset of insulitis and the development of overt diabetes (Chen et al., 2005). These findings suggest that diabetes onset might be associated with a reduction in Treg cell numbers and/or functions. An alternative, but not mutually exclusive, explanation is that the onset of diabetes is a result of the emergence of regulation-resistant effector T cells (Teff cells). Studies of Treg cell dynamics in human T1D patients have produced disparate results, ranging from reduced Treg cell frequency (Kukreja et al., 2002) or function (Brusko et al., 2005; Lindley et al., 2005) to no changes when comparing diabetic subjects to healthy control subjects (Putnam et al., 2005). Three independent investigations of NOD mice uniformly found an age-dependent decline in Treg cell function (Gregori et al., 2003; Pop et al., 2005; You et al., 2005). Two of these reports also demonstrated a reciprocal age-dependent increase in Teff cell resistance to regulation. However, data on the frequency of Treg cells in NOD mice are inconclusive at present. One study reported an age-dependent decline in the frequency of *Foxp3*-mRNA-expressing CD4$^+$ cells in the pancreatic lymph nodes (PLNs) and islets (Pop et al., 2005), whereas another found an increase in *Foxp3* mRNA at the time of diabetes onset compared to 6-week-old prediabetic control subjects (You et al., 2005).

Here, we report our study examining the population dynamics of CD4$^+$ Teff cells and Treg cells during the progression of T1D in NOD mice. Our investigation revealed a paradoxical increase of Treg cells in the PLN at the time of diabetes onset and demonstrated that disease progression was associated with a loss of Treg cell:Teff cell balance in the inflamed islets and concomitant reduction of CD25 and Bcl-2 expression in intra-islet Treg cells. Additionally, administration of IL-2 promoted Treg cell survival



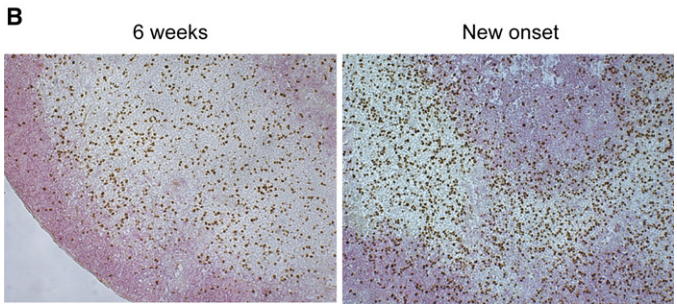


Figure 1. Preserved Treg Cell:Teff Cell Balance in PLN during T1D Progression

(A) Flow-cytometric analysis of the percentage of Foxp3$^+$ cells in the total CD4$^+$ T cell population of the PLNs of NOD mice. Representative histograms from an 8-week-old prediabetic NOD mouse and a new-onset-diabetic NOD mouse are shown.

(B) Immunohistochemistry of Foxp3 (brown) and B200 (pink) expression in the PLNs of 6-week-old prediabetic NOD mice and new-onset-diabetic NOD mice. Data represent five independent experiments with a total of 10–12 mice from each disease group.

and protected NOD mice from diabetes. This study demonstrates that cellular dynamics in local tissues play the key role in determination of the balance of immune homeostasis and disease progression. Our finding that IL-2 deficiency contributes to intra-islet Treg cell dysfunction and progressive breakdown of peripheral self-tolerance in the NOD mouse could have important implications for the use of IL-2-modulating therapies for the treatment of diabetes and other autoimmune diseases.

RESULTS

Preservation of Treg Cell:Teff Cell Balance in the PLN at Diabetes Onset

To determine whether the onset of diabetes in NOD mice reflects a general decline in Treg cells and an excess of autoreactive Teff cells, we examined the Treg cell:Teff cell balance in the PLN of the NOD mouse at different stages of disease progression in vivo. Unexpectedly, the Treg cell fraction of the total CD4$^+$ T cell population in the PLN did not decrease, but rather increased by 50%–70% at the time of diabetes onset (Figure 1A and Table S1, available online). No significant change in the percentage of Treg cells was found in distal inguinal LN (ILN) (Table S1). The absolute number of total Treg cells in PLN at the time of disease onset varied from mouse to mouse, mostly due to the variation in the cellularity of the PLN. However, the increase in the percentage of Treg cells at the time of disease onset was consistently reproduced in a large number of mice followed during a three-year study period, with the use of in situ detections via immunohistochemistry (Figure 1B) and immunofluorescence (see below) or more quantitative analyses via flow cytometry with the use of two different clones of anti-Foxp3 mAb conjugated with various fluorochromes.

Sustained Activation of PLN Treg Cells

To determine whether Treg cells present in the PLN at the time of diabetes onset were functionally suppressive, we examined the activation status of Treg cells. A higher fraction of PLN Treg cells showed reduced CD62L expression in newly diabetic mice when compared to Treg cells in 6-week-old prediabetic animals (Figure 2A), suggesting that Treg cells were more activated in the PLN at the time of disease onset (Huehn et al., 2004; Tang et al., 2004). Our previous work showed that Treg cells effectively prevent the interaction of Teff cells with dendritic cells (DCs) (Tang et al., 2006). Thus, we transferred CD4$^+$CD25$^-$CD62L^{hi} naive T cells from BDC2.5 TCR-transgenic mice and examined their interaction with endogenous DCs in newly diabetic and 6-week-old prediabetic mice by two-photon microscopy. Clustering and swarming of the islet-reactive BDC2.5 T cells, indicative of their interaction with endogenous DCs, was absent in newly diabetic mice, in sharp contrast with that found in the PLNs of 6-week-old prediabetic mice (Figure 2B, Movies S1 and S2). Consistently, proliferation of the adoptively transferred BDC2.5 T cells was markedly reduced at the time of diabetes onset (Figure 2C). Sections of the PLN were stained with mitotic marker Ki67, in addition to CD4 and Foxp3, to compare levels of the priming of endogenous Teff cells in the PLNs of young prediabetic and newly diabetic NOD mice. A marked reduction in the number of proliferating endogenous Teff cells (CD4$^+$Foxp3$^-$) in the PLN at the time of disease onset was evident, correlating with a concomitant increased number of CD4$^+$Foxp3$^+$ Treg cells (Figure 2D). These results suggest that the imbalance of Treg cells in the PLNs of newly diabetic NOD mice prevented naive effector cells from becoming activated when recruited to the PLN at this late time point in disease progression, although the reduced proliferation of Teff cells could also be contributed to reduced availability of islet antigens due to the reduction in β cell mass in overtly diabetic mice. It is important to point out that the increase in Treg cells was specific to the PLNs of the newly diabetic NOD mice (Table S1) and was not observed in chronically diabetic animals (data not shown), suggesting that the increase in Treg cells was dependent on the presence of residual islet autoantigens. Taken together, our results demonstrated an increased number and function of Treg cells in the PLN as a result of sustained Treg cell activation, leading to reduced Teff cell priming over the course of disease progression.

Loss of Treg Cell:Teff Cell Balance in the Islets

Expansion of Treg cells during chronic inflammation has been reported to be associated with disease resolution (Knoechel et al., 2005; Korn et al., 2007). However, our results showed a relative increase of Treg cells in the PLN with disease progression. This paradoxical observation prompted us to analyze Treg cell dynamics in the islets of NOD mice. Because leukocyte infiltration

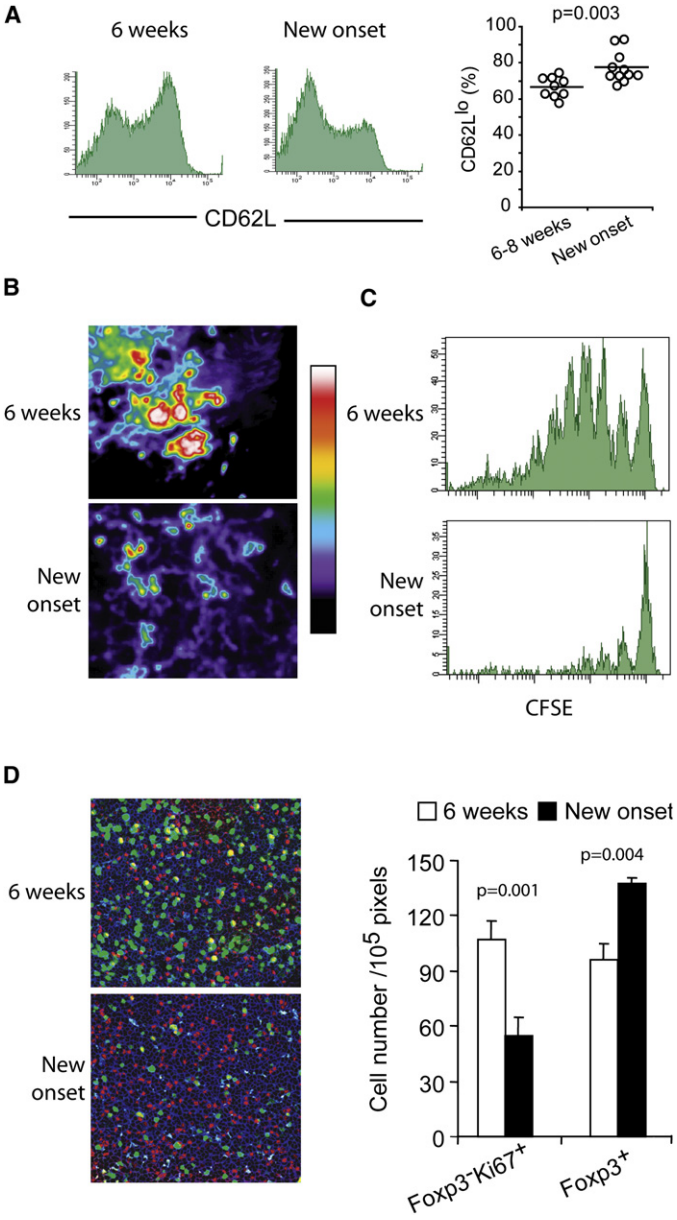


Figure 2. Preservation of Treg Cell Functions and Reduced Teff Cell Priming in the PLN at the Time of Disease Onset

(A) Flow-cytometric analysis of CD62L expression in $CD4^+$ $Foxp3^+$ cells in the PLNs of 6-week-old prediabetic NOD mice and new-onset-diabetic NOD mice. The graph on the right is a summary of four independent experiments comparing CD62L expression in $CD4^+CD25^+$ cells in the pancreatic LNs of young prediabetic (n = 9) and newly diabetic (n = 11) female NOD mice.

(B) $CD4^+CD62L^+CD25^-$ cells from BDC2.5 T cell receptor transgenic mice were purified by FACS, labeled with CFSE, and transferred to 6-week-old prediabetic NOD mice and to mice that were within three days of diabetes onset. The movement dynamics of transferred Treg cells in explanted PLNs were monitored with two-photon laser-scanning microscopy. The normalized average CFSE fluorescence intensity over a 30 min imaging period is illustrated in the "heat maps" shown. The large aggregates of strong fluorescence intensity represented by the yellow, red, and white colors indicates restricted movement of cell clusters due to antigen recognition, and the lower intensity represented by the blue, purple, and black colors indicates random movement in the absence of antigen recognition.

(C) In vivo proliferation of transferred $CD4^+CD62L^+CD25^-$ cells from BDC2.5 T cell receptor transgenic mice in PLNs of 6-week-old prediabetic mice or new-onset-diabetic mice were determined by CFSE dilution assay. Two representative histograms are shown.

(D) Proliferation of endogenous T cells in the PLNs of 6-week-old prediabetic NOD mice and new-onset-diabetic NOD mice was determined by costaining of PLN sections for the mitotic marker Ki67 (green), anti-CD4 (blue), and anti-Foxp3 (red). Representative micrographs are shown (left), and the average numbers of $Foxp3^-Ki67^+$ cells and $Foxp3^+$ cells in three randomly selected objective fields in the T cell zones of the PLNs are summarized (right; mean ± standard deviation [SD]; n = 3 mice). Results in each panel represent two to four independent experiments.

and tissue destruction progress asynchronously in different islets (Anderson and Bluestone, 2005), confocal microscopic analyses of immunofluorescent antibody-stained pancreatic sections were performed to characterize the $Foxp3^+CD4^+$ Treg cell population within individual islets (Figure S1). Such analyses revealed marked heterogeneity in the percentage of Treg cells, from 5% to 30%, in individual islets. Although the total numbers of Treg cells in each islet increased as the number of intra-islet Teff $CD4^+$ cells expanded (Figure S2), the percentage of Treg cells dropped precipitously, revealing a striking inverse relationship between the magnitude of infiltration and the percentage of Treg cells in the individual islets (Figure 3A). The reduction in Treg cell percentages was most pronounced in islets from prediabetic mice that exhibited mild (grade 1) or moderate (grade 2) insulitis (less than 500 $CD4^+$ cells per islet section), suggesting that the relative loss of Treg cells in the islets preceded the escalation of inflammation and islet destruction. These results demonstrated an uncoupling of the PLN and the pancreas in terms of Treg cell:Teff cell balance over the course of the disease and suggested that the progressive dysregulation of immune homeostasis in islets led to β cell destruction and development of diabetes.

Differential proliferation, trafficking, and/or survival of Teff cells and Treg cells could explain the loss of Treg cell:Teff cell balance in the islets. To compare the proliferation rate of intra-islet Teff cells and Treg cells, we stained pancreatic sections for Ki67 and determined the percentages of proliferating Teff cells ($CD4^+Foxp3^-Ki67^+$) and Treg cells ($CD4^+Foxp3^+Ki67^+$) in individual islets. The total numbers of proliferating Teff cells and Treg cells increased as the intra-islet infiltrate expanded (Figures S3 and S4). A direct comparison of $Foxp3^+$ and $Foxp3^-$ cell proliferation rate in the same islets demonstrated that higher percentages of Treg cells entered mitosis in most of the islets examined independent of the age of the mice (Figure 3B). Flow-cytometry analysis of Ki67 expression by intra-islet Treg cells and Teff cells also demonstrated that a higher fraction of Treg cells were in cycle as compared to Teff cells (data not shown). Thus, the relative decline of Treg cells seen in islets with increased infiltration was not due to a reduction in Treg cell proliferation compared to that of Teff cells.

Defective Survival of Intra-Islet Treg Cells

We tested whether a defect in intra-islet Treg cell survival could explain the relative loss of Treg cells. Because CD25 expression

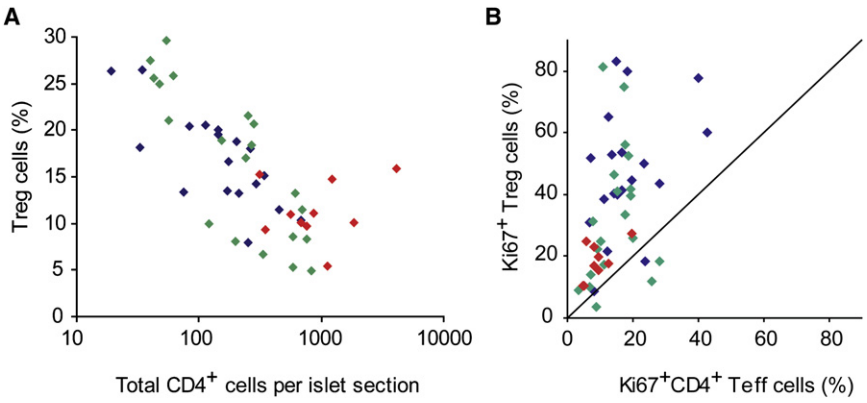


Figure 3. Dynamics of Teff Cells and Treg Cells in the Islets of NOD Mice during Diabetes Progression

(A) Numbers of $CD4^+$ and $Foxp3^+$ cells in individual islets were quantified by manual counting of frozen pancreatic sections strained with immunofluorescence-labeled antibodies. The percentages of $Foxp3^+$ Treg cells among $CD4^+$ T cells in individual islet sections are plotted against the total numbers of $CD4^+$ cells in the corresponding islet sections. Correlation analysis revealed a significant inverse correlation (Spearman $r = -0.707$) between the numbers of total $CD4^+$ T cells per islet section and the percentage of Treg cells in the corresponding islet ($p < 0.0001$).

(B) Numbers of $Ki67^+CD4^+Foxp3^-$, $Ki67^-CD4^+Foxp3^-$, $Ki67^+CD4^+Foxp3^+$, and $Ki67^-CD4^+Foxp3^+$ cells in individual islets were quantified as described in panel (A) legend. Percentages of proliferating ($Ki67^+$) Treg cells versus proliferating Teff cells in islets were calculated and plotted. For both panels, blue symbols represent islets from 6- to 8-week-old NOD mice, green symbols represent those from 10- to 12-week-old mice, and red symbols represent those from mice with recent diabetes onset.

is essential for Treg cell survival in response to IL-2 in the periphery (D'Cruz and Klein, 2005; Fontenot et al., 2005a; Tang et al., 2003), we measured CD25 expression in $Foxp3^+$ cells in ILNs, PLNs, and islets. Greater than 85% of Treg cells in the ILNs of prediabetic and newly diabetic NOD mice expressed CD25, similar to that reported for C57BL/6 mice (Fontenot et al., 2005b). Treg cells in the PLN exhibited a moderate reduction of CD25 expression as compared to Treg cells in the ILN (Figure 4A). In contrast, only ~50% of intra-islet Treg cells expressed CD25 (Figure 4A). More strikingly, the mean fluorescent intensity of CD25 staining in the intra-islet $Foxp3^+$ cells was 20% of that of their ILN counterparts (Figure 4B). The reduced CD25 expression in intra-islet Treg cells was evident in young prediabetic mice, coinciding with the shift in Treg cell:Teff cell balance in the islets at the early phase of the pathogenic response (Figure 3A). $Foxp3^+$ cells from other inflammatory sites, such as lacrimal and salivary glands, also exhibited lower CD25 expression (Figure S5), demonstrating that the loss of CD25 expression in Treg cells at inflammatory sites was not an islet-specific phenomenon, but rather a general deficiency in the NOD mice.

These data, combined with the increased proliferation of these cells, are consistent with the notion that intra-islet Treg cells are more prone to apoptosis. However, current methods for the measuring of T cell apoptosis, including TUNEL, annexin V, or caspase 3 staining, were not sensitive enough for in vivo analysis because of the efficient removal of apoptotic cells by the scavenging system (Krysko et al., 2006). Therefore, we examined the expression of an intracellular biochemical marker of IL-2-driven cell survival, Bcl-2. Bcl-2 is an anti-apoptotic effector molecule (Miyazaki et al., 1995), and its loss occurs at an early stage of programmed cell death before the loss of cytoplasm-membrane integrity and the subsequent sensing by the scavenging system. Bcl-2 was expressed at similar high amounts in both Treg cells and Teff cells in inguinal and pancreatic LNs (Figure 4C). In contrast, Bcl-2 expression was significantly lower in islet-infiltrating T cells, suggesting that levels of IL-2 were limiting in the inflamed tissue. Most importantly, the reduction in Bcl-2 expression was more severe in intra-islet Treg cells (66% reduced as compared to LN-resident Treg cells) than in intra-islet Teff cells (36% reduced as compared to LN-resident Teff cells) (Figure 4D). We have examined Bcl-2 expression in mice of a wide age range (from 8 to 25 weeks old), and we found that the loss of Bcl-2 in intra-islet Treg cells was evident at the earliest time examined and persisted through diabetes onset (data not shown).

To directly determine if there is a local deficiency of IL-2 production in the inflamed islets relative to LNs and spleens, we purified $CD4^+$ T cells and measured IL-2 mRNA expression by real-time RT-PCR. Without in vitro stimulation, IL-2 mRNA was present in all tissues at a low amount just above the detection threshold (Figure 4E). IL-2 mRNA increased and become readily detectable after three hours of stimulation with anti-CD3 and anti-CD28. $CD4^+$ cells from islets produced significantly lower amounts of IL-2 mRNA as compared to $CD4^+$ cells from spleen or ILN (Figure 4E). Together, these findings suggest that a defect in the survival of Treg cells, secondary to a deficiency in IL-2 production, accounts for the selective loss of the Treg cell:Teff cell balance in the islets.

Low-Dose IL-2 Promotes Treg Cell Survival and Protects NOD Mice Against Diabetes

The hypothesis that the selective loss of the Treg cell:Teff cell balance in the inflamed islets is due to enhanced apoptosis of intra-islet Treg cells as a consequence of local IL-2 deficiency is supported by previous studies demonstrating that the binding of IL-2 to CD25 and the subsequent STAT5 signaling are essential for the survival of $Foxp3^+$ Treg cells in the periphery (D'Cruz and Klein, 2005; Fontenot et al., 2005a; Furtado et al., 2002). In addition, IL-2 is one of the most potent inducers of CD25 and Bcl-2 expression in T cells (Shi et al., 2001); thus, the loss of CD25 and Bcl-2 strongly suggests an IL-2 deficiency. Further, several publications have reported a relative IL-2 deficiency in NOD mice, a defect mapped to the *Idd3* locus on chromosome 3 (Denny et al., 1997; Yamanouchi et al., 2007). To test our hypothesis that enhanced intra-islet Treg cell apoptosis secondary to an IL-2 deficiency led to islet destruction, we initiated studies to determine whether correction of the IL-2 deficiency would improve Treg cell survival and confer disease protection. One-week treatment of prediabetic NOD mice with a cocktail of 5 μg recombinant IL-2 and 50 μg IL-2 mAbs, a regimen previously

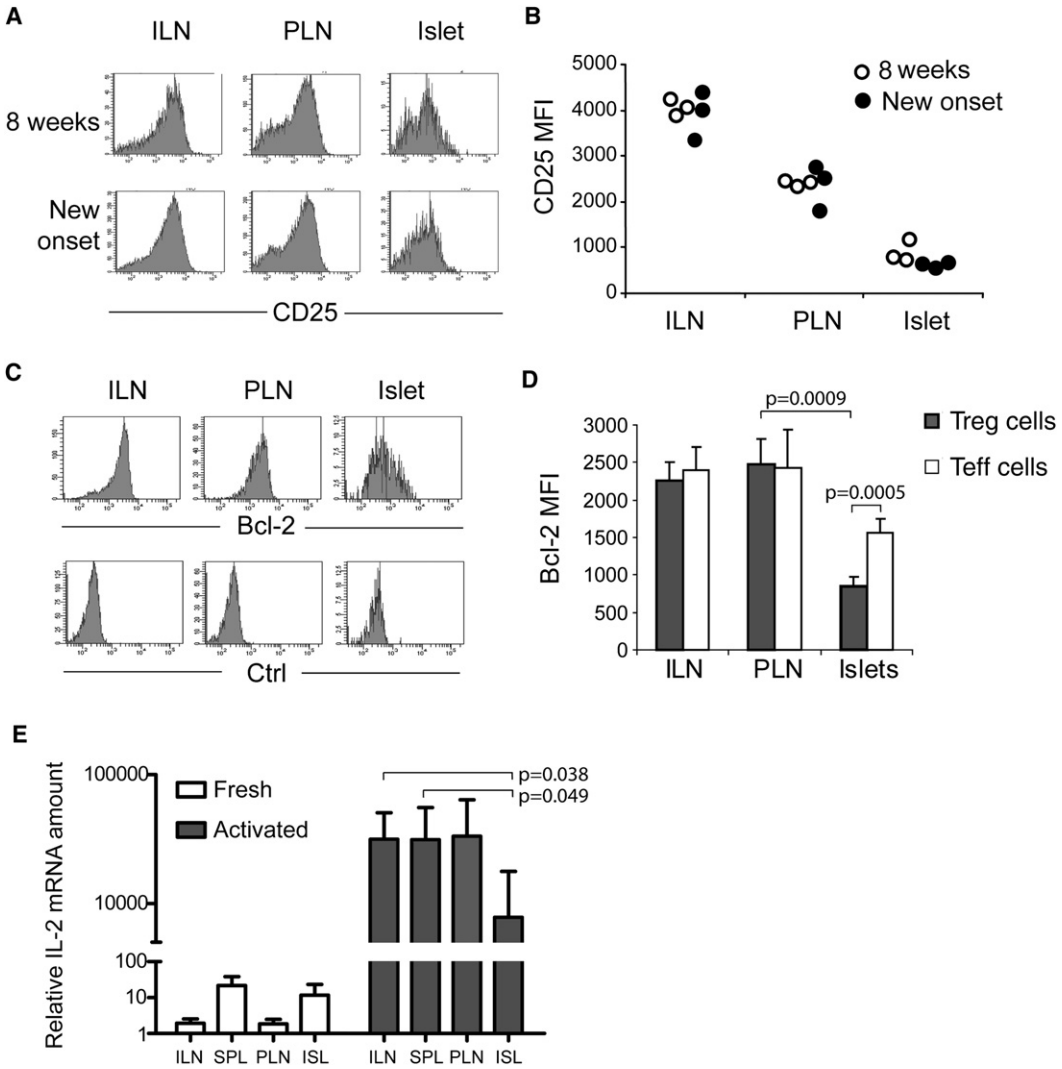


Figure 4. Loss of CD25 and Bcl-2 Expression in Intra-Islet Treg Cells

(A and B) Flow-cytometric analyses of CD25 expression in $CD4^+Foxp3^+$ cells. Representative CD25 versus Foxp3 dot plots of $CD4^+$ cells in ILN, PLN, and islets are shown in (A), along with a graph summarizing the mean fluorescence intensities of CD25 in $CD4^+Foxp3^+$ cells (B). The open symbols represent 8-week-old mice, and filled symbols represent mice with recent diabetes onset. Results are representative of four independent experiments.

(C and D) Flow-cytometric analysis of Bcl-2 expression in $CD4^+Foxp3^+$ cells. Representative Bcl-2 (top) and isotype control staining (bottom) histograms of $CD4^+Foxp3^+$ cells in ILN, PLN, and islets are shown in (C), along with a bar graph summarizing the mean fluorescence intensities of Bcl-2 expression in $CD4^+Foxp3^+$ cells (D; mean ± SD, n = 4). Results are representative of five independent experiments.

(E) Real-time RT-PCR analysis of IL-2 mRNA in various lymphoid organs and islets. Tissue samples from prediabetic NOD female mice were assayed individually (mean ± SD; n = 4). A student t test was performed to determine the statistical significance of the difference, and p values for the significantly different sample pairs are shown on the graph.

shown to selectively expand Treg cells without increasing $CD8^+$ memory T cells in C57BL/6 mice (Boyman et al., 2006), led to a marked increase in CD25 expression in Treg cells (Figure 5A) and a systemic increase in the percentage of Treg cells (Figure 5B). At this dose, the treatment increased CD25 expression on $CD4^+Foxp3^-$ and $CD8^+$ T cells of prediabetic NOD mice (Figures 5C and 5D). In addition, a substantial expansion of NK cells were observed in LN and spleen and among islet infiltrates (Figure 5E). Notably, one week treatment at this dose in 10-week-old female prediabetic mice rapidly precipitated diabetes and even death in some animals (Figure 5F). Thus, high-dose IL-2 treatment enhanced functions of pathogenic Teff cells and the number of Treg cells, with a net result of accelerated autoimmune tissue destruction.

Because Treg cells constitutively express CD25, a lower dose of IL-2 treatment might selectively act on Treg cells with minimal effect on $CD4^+$ or $CD8^+$ Teff cells. Using careful titration, we found that one tenth of the dose used in the experiments shown in Figure 5 led to a moderate increase in Treg cell percentages (Figures 6A and 6D), correlating with an increase in their CD25 and Bcl-2 expression (Figures 6A–6C). At this dose, a mild increase in $CD4^+$ ($Foxp3^-CD25^{hi}$) or $CD8^+$ ($CD25^{hi}$) Teff cells

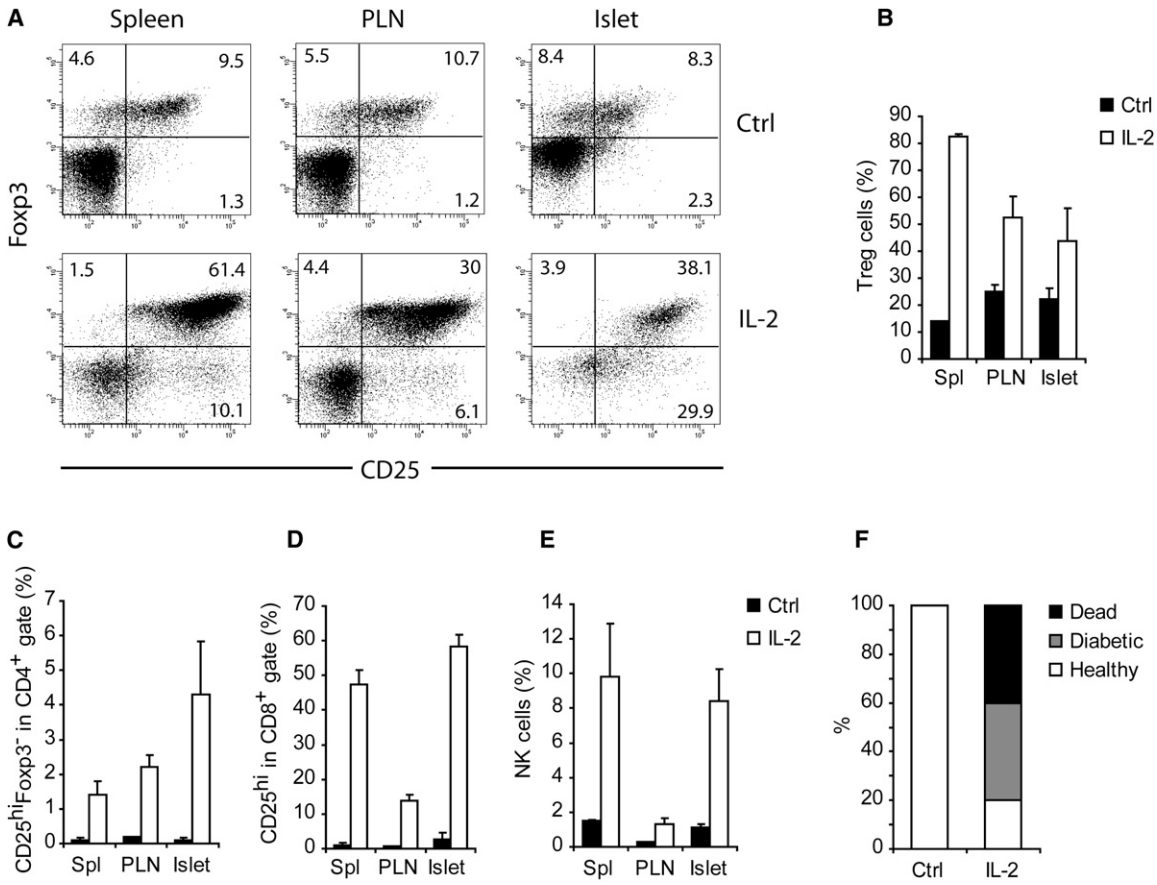


Figure 5. Effect of High-Dose IL-2 Treatment on Prediabetic NOD Mice

A cohort of 10-week-old female NOD mice were treated with daily intraperitoneal injections of 5 μg IL-2 and 50 μg of anti-IL-2 complex or irrelevant rat IgG as control for five consecutive days (n = 3 per condition). One week after the initiation of the treatment, the composition of the spleen, PLN, and intra-islet infiltrates were analyzed by flow cytometry.

(A) Dot plots displaying Foxp3 and CD25 expression in $CD4^+$ cells in control- (top) and IL-2 complex-treated (bottom) mice.

(B–E). (B) Bar graphs summarizing the frequency of Treg cells and (C) $CD25^{hi}Foxp3^-$ Teff cells among $CD4^+$ cells, (D) the frequency of $CD25^{hi}$ cells among $CD8^+$ cells, and (E) the percentage of NK cells among all $CD45^+$ leukocytes are shown (mean ± SD; n = 3). The differences between control and IL-2-treated samples in all organs analyzed were found to be significant ($p < 0.05$) by the student t test analysis. Results are representative of three independent experiments.

(F) A separate cohort of 10-week-old female NOD mice were treated with control rat IgG or IL-2 complex as in (A) (n = 10 per group). The percentage of mice who died, became diabetic, or remained healthy one week after the treatment is summarized. Result is representative of three separate experiments.

was observed (Figures 6E and 6F), and there was no marked effect on NK cells (Figure 6G). To determine the long-term effect of the low-dose IL-2 therapy on diabetes development, a cohort of female NOD mice was treated from 10 to 20 weeks of age. Control mice were treated with an anti-human HLA-Bw6 rat mAb or saline for the same period. Low-dose IL-2 treatment prevented diabetes development in the majority of the mice (Figure 7A). Similar results were observed when mice were treated with recombinant human IL-2 from 5 to 20 weeks of age (Figure 7B). These data were confirmed by histological examination of the mice that remained free of diabetes at the end of the experiment. There was a marked reduction in the severity of insulitis in the mice that received IL-2 treatment (Figure 7C). Taken together, our results suggest that the lower expression of CD25 in intra-islet Treg cells and their reduced survival were not due to defects intrinsic to NOD Treg cells. Rather, the reduced availability of IL-2 from the Teff cells is most likely the root cause of the progressive loss of Treg cell:Teff cell balance in the islet leading to β cell destruction.

DISCUSSION

In this study, we demonstrated that T1D progression in the NOD mouse was associated with a progressive loss of Treg cell:Teff cell balance in the inflamed islets but not in the PLN. Intra-islet Treg cells expressed reduced amounts of CD25 and Bcl-2 relative to the Treg cells in the PLN, suggesting that the Treg cell:Teff cell imbalance was due a defect in intra-islet Treg survival. We further demonstrated that IL-2 treatment of NOD mice restored CD25 expression in intra-islet Treg cells and led to diabetes prevention.

It is well established that Treg cells control the progression of T1D in the NOD-mouse model (Chen et al., 2005; Salomon et al., 2000). However, the precise temporal and anatomical basis of

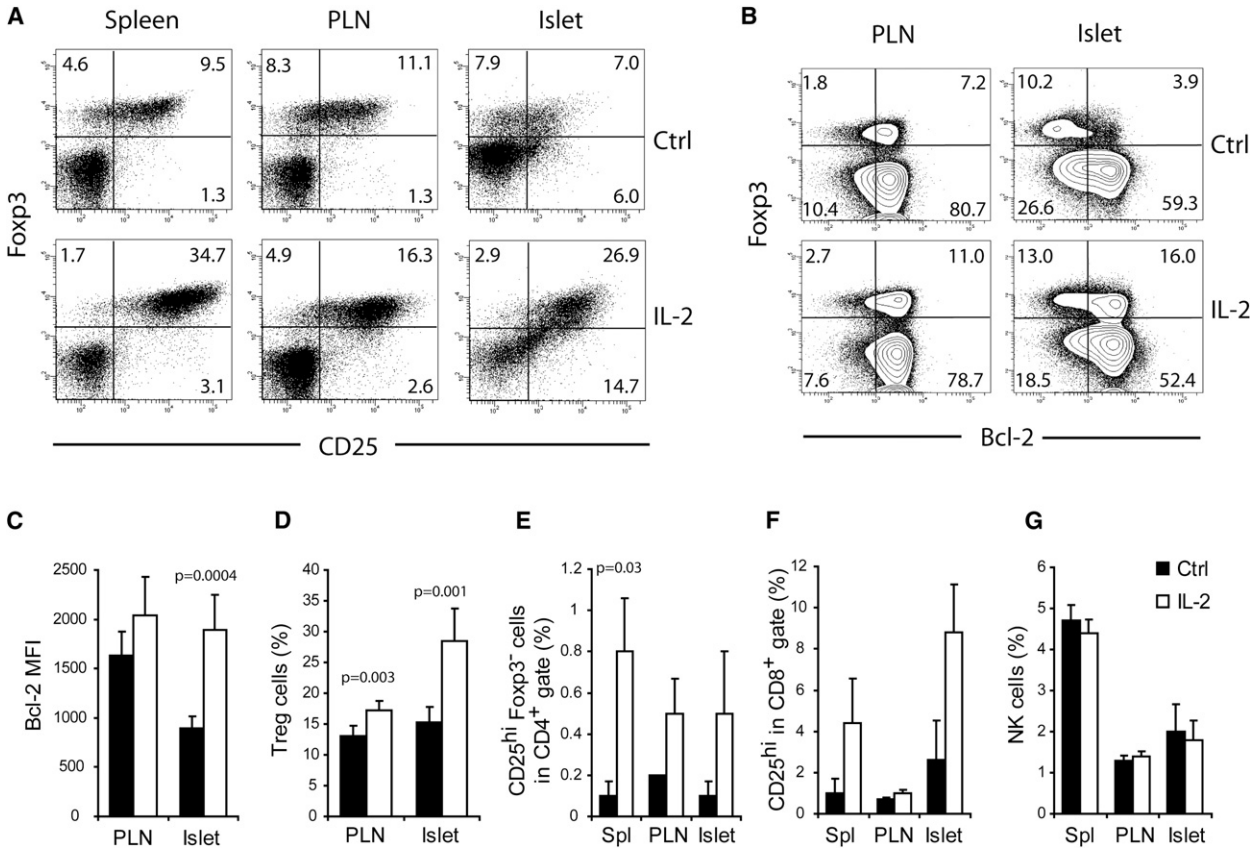


Figure 6. Low-Dose IL-2 Therapy Restores Treg Cell:Teff Cell Balance

A cohort of 10-week-old female NOD mice were treated with daily injections of 0.5 μg IL-2 and 5 μg anti-IL-2 complex or irrelevant rat IgG as control for five consecutive days. The expression of (A) CD25 and of (B and C) Bcl-2 in Treg cells in the PLN and the islets was determined by flow cytometry. Percentages of (D) Treg cells, (E) $Foxp^{-}CD25^{hi}$ $CD4^{+}$ Teff cells, (F) $CD25^{hi}$ $CD8^{+}$ Teff cells, and (G) NK cells are summarized (mean ± SD; n = 5 for [C], [D], and [G]; n = 3 for [E] and [F]). p values for the samples that showed significant differences between control and IL-2-treated mice are indicated in the graphs, and those for all others were not found to be significantly different by student t test ($p > 0.05$). Results are representative of at least three independent experiments.

Treg cell control is not clear, especially in regards to the relevant role of the PLN versus the inflamed islets as the major site of immune regulation (Bour-Jordan et al., 2004; Chen et al., 2005; Tang and Bluestone, 2006). In this study, we demonstrate that NOD Treg cells mount an appropriate response to tissue destruction in the PLN by expanding, acquiring an activated phenotype, and infiltrating inflamed islets. This attempt to "control" disease was also manifested within the islets, given that a high fraction of the Treg cells continue to divide within the pancreatic islet tissue, suggesting that further activation occurred at the site of inflammation. In fact, quantitative in vivo analysis of the Treg cell:Teff cell balance in individual islets revealed a high frequency of Treg cells in early islet infiltration in young prediabetic mice, similar to what has been observed in other settings, including models of tumor, infectious disease, and other autoimmune diseases (Aluvihare and Betz, 2006; Belkaid et al., 2006; Munn and Mellor, 2006; Rouse et al., 2006; Sakaguchi et al., 2006; Waldmann et al., 2006). However, in NOD mice, Treg cells failed to survive in the tissue long term, which was probably due to the limited availability of IL-2.

The importance of IL-2 in the maintenance of Treg cell homeostasis and suppression of T1D has been suggested by IL-2-neutralization studies (Setoguchi et al., 2005). Genetic-mapping studies have demonstrated that the NOD *Idd3* allele contributes to an IL-2 defect in the NOD mice (Denny et al., 1997). More recently, an elegant study further demonstrated that the NOD *idd3* allele led to systemic reduction in Treg cell frequency and higher mortality due to diabetes in a $CD8^{+}$ T cell-receptor-transgenic NOD model (Yamanouchi et al., 2007). Our findings in this study further illustrate that in untreated NOD mice, there is a selective defect in Treg cell survival in inflamed tissues. The survival defect was observed in mice as young as 6 weeks of age, supporting the notion that this phenotype is most likely genetically encoded. The reduced expression of IL-2 and IL-2-regulated genes such as *CD25* and *Bcl-2* in Treg cells suggests that the Treg cell survival defect was secondary to an IL-2 deficiency. The local inflammatory milieu in the islets might exacerbate the IL-2 shortage by further inhibiting IL-2 expression (Villarino et al., 2007), competing for IL-2 by activated Teff cells, and inducing the cleavage of CD25 via matrix metalloproteases induced by local inflammation (Sheu et al., 2001). Thus, the genetically-encoded inborn IL-2 deficiency in the NOD mice might be more pronounced in inflamed tissues, thus compromising Treg cell survival locally. Common γ-chain-binding cytokines such as IL-4,

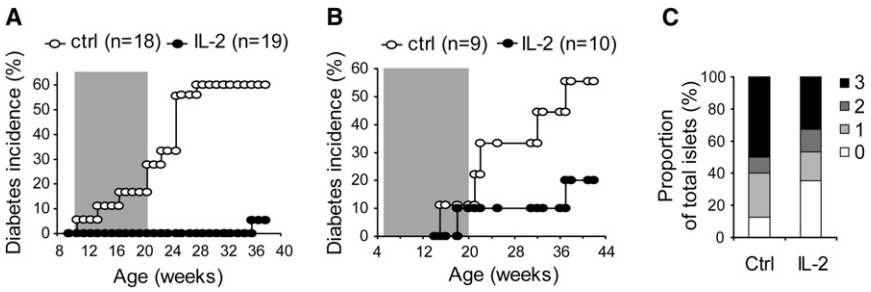


Figure 7. Low-Dose IL-2 Therapy Prevents Diabetes

(A and B) Diabetes progression in NOD mice treated with (A) 0.5 μg IL-2 and 5 μg anti-IL-2 complex or (B) recombinant human IL-2. Control mice received saline. Shaded areas inside the graphs indicate the duration of the treatments; between 10–20 weeks for mice indicated in (A) and between 5–20 weeks for mice indicated in (B). p values between the control and the IL-2-treated groups are 0.0002 for the experiment depicted in (A) and 0.11 for the experiment depicted in (B).

(C) Severity of intra-islet infiltration in mice that remain free of overt diabetes at the end of the experiment depicted in (B) was evaluated histologically, and the percentage of islets with no infiltration (0), peri-insulitis (1), moderate insulitis with less than 50% islet area infiltrated (2), and severe insulitis with greater than 50% islet area infiltrated (3) were determined and plotted. Data for the saline-treated control group represent 40 islets from four mice, and data from the IL-2-treated group represent 341 islets from eight mice.

IL-7, and IL-15 can help to sustain Treg cell survival in vitro through the provision of anti-apoptosis signals (Pandiyan et al., 2007). It is thus possible that deficiency in these cytokines could also contribute to the demise of Treg cells in the inflamed islets. Finally, it should be noted that the deficiency in Treg survival might be compounded by an independent genetic defect in Bcl-2 expression in NOD mice (Garchon et al., 1994). In fact, overexpression of Bcl-2 in T cells and B cells alleviated insulitis and conferred diabetes protection (Rietz et al., 2003).

Similar to intra-islet Treg cells, Treg cells isolated from inflamed lacrimal and salivary glands in the NOD mice also expressed markedly reduced CD25. Several published reports in various disease models demonstrated that tissue-infiltrating Treg cells could be readily identified by their surface expression of CD25 and that the cells were functional in the control of local inflammation (Belkaid et al., 2002; Yu et al., 2005). Thus, loss of CD25 is not a general characteristic of Treg cells in inflamed tissues. It remains to be determined whether the local IL-2 deficiency and Treg cell imbalance observed in this study were restricted to sites of autoimmune inflammation or reflected a more general defect of Treg cells in the NOD mouse. Examination of CD25 expression in Treg cells in other inflammatory settings in the NOD mice, such as microbial infection, would help to clarify this issue.

The polarized effect of high- and low-dose IL-2 therapy on autoimmune response observed in this study is striking and highlights the pleiotropic effect of this cytokine. Although IL-2 has an indispensable role in Treg cell homeostasis, it was originally discovered as a T cell growth factor and activator of cytotoxic lymphocytes (Taniguchi et al., 1983). IL-2 has been used in the clinic since the mid-1980s as an immune-boosting cancer therapy, with limited success. Part of the limitations in IL-2 cancer therapy might be due to the expansion of Treg cells (Wei et al., 2007). We observed that a regimen of multiple, low-dose injections of IL-2 selectively supported the survival and growth of Treg cells over that of Teff cells, whereas a high-dose regimen led to rapid Teff cell expansion and disease onset. In light of this result, it is interesting to point out that a high bolus dose of IL-2 was more efficacious than a low dose in the treatment of renal cell carcinomas (Fisher et al., 2000; Yang et al., 2003). Together, the results suggest that the in vivo effect of IL-2 can vary widely depending on the dosing regimen, the amount of endogenous IL-2, and the numbers of activated CD4 and CD8 Teff cells, NK cells, and Treg cells in the host. Thus, an optimal IL-2 treatment regimen could be difficult to predict for a heterogeneous patient population, and adjunct therapy will be needed to ensure the desired outcome. For example, the combination of IL-2 treatment with Teff cell-depleting treatments such as anti-CD3 or Rapamycin could prevent potential disease exacerbation and help to restore long-term self tolerance in an autoimmune setting (Chatenoud, 2003; Rabinovitch et al., 2002). In contrast, in a cancer setting, IL-2 treatment in conjunction with Treg cell depletion might be more effective than IL-2 monotherapy for the induction of tumor regression.

Three independent studies in the NOD mice demonstrated that diabetes progression is associated with the acquisition of regulation resistance by Teff cells over time (Gregori et al., 2003; Pop et al., 2005; You et al., 2005). Our results extend these findings, suggesting that the development of diabetes in the face of increasing frequencies of Treg cells might reflect this increased Teff cell resistance. The protracted disease course in the NOD mice might reflect the time needed for regulation-resistant Teff cells to emerge and accumulate to sufficient numbers under the constant control of Treg cells. Furthermore, the regulation-resistant phenotype could be linked to the reduced IL-2 production of these Teff cells. We previously demonstrated that Treg cells expand in the presence of strong CD28 costimulation, and large amounts of IL-2 can effectively prevent and even reverse diabetes (Tang et al., 2004). These Treg cells survive long-term (greater than 50 days) in recipient mice and are less dependent on B7 costimulation and IL-2 from the hosts (Q.T. and J.A.B., unpublished observations). Thus, the heightened regulation resistance and unfavorable survival environment for Treg cells found in the NOD mice can be overcome by Treg cell therapies if appropriate Treg cell-preparative regimens are used.

Immune deficiency is often associated with autoimmunity in mice and man (Arkwright et al., 2002; Dupuis-Girod et al., 2003; Horak et al., 1995; Mombaerts et al., 1993), and in the NOD mouse, immune stimulation can protect mice against diabetes (Qin and Singh, 1997; Sharif et al., 2001). Our findings provide one possible explanation for these paradoxical observations, suggesting that normal Treg cell homeostasis and a healthy balanced immune system depend ultimately on a robust Teff cell response. IL-2 production by activated Teff cells expands and sustains Treg cells, which in turn feed back to suppress the Teff cell response and maintain normal immune homeostasis. Disruption of this crosstalk can lead to the dysregulation of the Treg cell:Teff

cell balance and contribute to the development of autoimmune diseases in the NOD mice. The effective control of diabetes with low-dose IL-2 treatment leads to an intriguing suggestion that, in some instances, immune stimulation rather than immunosuppression could be an effective approach for the treatment of autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

Female NOD (Taconic, Germantown, NY) and NOD.BDC2.5 TCR-transgenic mice were housed and bred under specific pathogen-free conditions at the UCSF Animal Barrier Facility.

Treatment with IL-2

Eight- to ten-week-old female NOD mice were treated with an IL-2 and an anti-IL-2 complex. A 100 μl PBS solution containing 0.5 to 5 μg recombinant mouse IL-2 (eBioscience, San Diego, CA, USA) and 5 to 50 μg rat anti-mouse IL-2 (clone JES6-12A1, R & D Systems, Minneapolis, MN) was injected into the peritoneal cavity of each mouse every day for five days. Control mice were injected with either saline or 50 μg irrelevant mAb (rat anti-human HLA-Bw6). For diabetes-prevention studies, female NOD mice were treated for five consecutive days followed by twice-per-week maintenance treatments for a total duration of 10 weeks. Dosings for each specific experiment are indicated in the Results section. In separate experiments, human recombinant IL-2 was used at a dose of 25,000 international units three times per week for the duration as indicated in the results section.

Flow Cytometry

Islets were purified, with standard collagenase protocols followed as described (Tang et al., 2004), and dissociated by incubation with a nonenzymatic solution (Sigma, St, Lois, MO, USA) followed by trituration per the manufacturer's instructions. LN cells were made into a single cell suspension by mechanic disruption or as described for islet cells. The following antibodies were used to stain the cells: FITC- or PerCP-labeled anti-CD45 (LCA; BD PharMingen, San Diego, CA), Phycoerythrin (PE)- or FITC-labeled anti-CD25 (clone PC61; eBioscience), PerCP- or Alexa 700-labeled anti-CD4 (BD PharMingen, San Diego, CA, USA), FITC- or Alexa 700-conjugated anti-CD8 (Invitrogen, Carlsbad, CA, USA), and biotinylated anti-NKG2D, followed by Quantumdot 605-labeled streptavidin (Invitrogen). The cells were then fixed, permeabilized, and stained with APC-labeled Foxp3 antibody with the use of a Foxp3 labeling kit per manufacturer's instructions (eBioscience). Cell-surface staining with anti-CD25 was omitted in some experiments in favor of intracellular staining with PE anti-Bcl-2 (BD) and/or FITC anti-Ki67 (BD). Flow-cytometric analyses were performed on an LSRII flow cytometer with FACS Diva software (Becton Dickenson, San Jose, CA, USA).

Multicolor Immunofluorescent Labeling and Confocal Microscopy

The PLN and pancreata were harvested and frozen in OCT. Six micron cryosections were fixed in ethanol and incubated with rabbit anti-mouse Foxp3 (provided by R. Khattri) and biotinylated mouse monoclonal anti-Ki67, followed by goat anti-rabbit Alexa 555 (Invitrogen), streptavidin FITC, and anti-CD4 Alexa 647 (eBioscience). The resulting fluorescent-staining pattern was detected and acquired with an SP2 laser-scanning confocal microscope (Leica, Wetzlar, Germany), and postacquisition analyses were performed with the aid of the Metamorph software (Universal Imaging, Downingtown, PA, USA). To quantify the numbers of $CD4^+Foxp3^+$, $CD4^+Foxp3^-$, and $Ki67^+$ cells within each of the individual islets, a manual counting method was used with the aid of the Metamorph software. High-resolution digitally zoomed images were used to ensure accuracy in counting. Numbers obtained from contiguous nonoverlapping regions of a single islet were added to derive a total number of Teff cells and Treg cells in each islet. To compare the number of Treg cells and proliferating Teff cells in the PLNs of 6-week-old prediabetic NOD mice and recently diabetic NOD mice, the numbers of $CD4^+Foxp3^+$ cells and $CD4^+Foxp3^-Ki67^+$ cells in nonoverlapping 10^5 pixel (one objective field) areas in the T cell zones (indicated by continuous CD4 staining) were determined by manual counting with the aid of the Metamorph software.

Two-Photon Laser Scanning Microscopy Analysis

FACS-purified $CD4^+CD62L^{hi}CD25^-$ cells from NOD.BDC2.5 TCR-transgenic mice were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) before retro-orbital injection into 6-week-old prediabetic or recently diabetic NOD mice ($3.5–5 \times 10^6$ per mouse). Two-photon microscopy was performed with a custom resonant-scanning instrument as described (Tang et al., 2006). In brief, PLN were excised, immobilized to coverslips with the hilum of the LN facing away from the objective. During imaging, the LNs were maintained in 36°C RPMI medium bubbled with 95% O_2/5% CO_2, and they were imaged through the capsule in a region distal to the hilum. For time-lapse image acquisition, images of up to 50 xy planes with 2–5 μm z spacing were acquired every 30 s for 30 to 60 min. Postacquisition data analyses were performed with the aid of the Metamorph software (Universal Imaging).

Quantification of IL-2 mRNA by Real-Time RT-PCR

Islet single-cell suspension was made as described in the section regarding flow cytometry. $CD4^+$ T cells from islets, ILN, PLN, and spleen were purified by positive selection with autoMACS. Samples were kept separate per individual mice, and two thirds of the purified $CD4^+$ cells from each sample were lysed immediately after purification in Trizol (Invitrogen) for RNA isolation. The remaining one third were stimulated in vitro with plate-bound anti-CD3 and anti-CD28 (1 μg per ml each) for 16 hr before RNA isolation by Trizol. The same amount of RNA was used to make cDNA with the use of oligo dT primers and a reverse transcription kit per manufacturer's instructions (SuperScript III Reverse Transcription kit, Invitrogen). Then, 10% of each of the cDNA reactions was used as a template for real-time PCR. The sequences for PCR primers are: IL-2 forward, 5′–AAAAGCTTTCAATTGGAAGATGCTG–3′; IL-2 reverse, 5′–TTGAGGGCTTGTTGAGATGA–3′; β-actin forward, 5′–AAGTGTGACGTTGACATCCGTAA–3′; and β-actin reverse, 5′–TGCCTGGGTACATGGTGGTA–3′. PCR was carried out with a Bio-Rad iQ5 Real-Time PCR Detection System (Hercules, CA), and Cyber Green (Invitrogen) was used for the quantitative detection of amplified DNA. Signals from the β-actin reactions were used to normalize the signals in the IL-2 PCR reactions of the same cDNA sample to derive the relative IL-2 mRNA level for each sample.

Statistical Analysis

To determine the relationship between the size of intra-islet infiltrates and the percentage of Treg cells in individual islets, correlation analysis was performed with the aid of GraphPad Prism software (San Diego, CA, USA). The Spearman r value was calculated to determine the types of correlation (0 = no correlation, 0 to 1 = positive correlation, and −1 to 0 = inverse correlation), and a two-tailed p value was used to determine the significance of the correlation. To determine the effect of IL-2 treatment on diabetes progression, diabetes-free fractions in control groups and in those treated with IL-2 over time were calculated with the Kaplan-Meier method, and the Log-rank test was used to compare the differences between the treatment groups. A two-tailed student t test was used to determine the significance of the two different treatment groups as specified in the Results section.

SUPPLEMENTAL DATA

Supplemental Data include five figures, one table, and two movies and can be found with this article online at http://www.immunity.com/cgi/content/full/28/5/687/DC1/.

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